Medical and Pediatric Oncology 30:228–232 (1998)

Molecular Monitoring of Tumor Cell Contamination in Leukapheresis Products From Stage IV Neuroblastoma Patients Before and After Positive CD34 Selection

A. Tchirkov, MD,[1,2*] J. Kanold, MD,[1] M. Giollant, PhD,[2] P. Halle-Haus, PhD,[1] M. Berger, MD,[1] C. Rapatel, MD,[1] P. Lutz, MD,[3] C. Bergeron, MD,[4] D. Plantaz, MD,[5] J.-P. Vannier, MD,[6] J.-L. Stephan, MD,[7] M. Favrot, MD,[8] P. Bordigoni, MD,[9] P. Malet, MD,[2] G. Briançon, TC,[2] and F. Deméocq, MD, PhD[1]

Background. Autologous peripheral blood stem cells (PBSCs) are frequently used to reconstitute hematopoiesis following administration of megatherapy in children with advanced stage IV neuroblastoma. Some centers prefer the use of autografts enriched for CD34+ progenitor cells because the positive selection procedure is believed to reduce indirectly tumor cell contamination.

Procedure. In this study, we monitored the efficiency of tumor cell purging following CD34 selection in PBSCs from seven patients with advanced neuroblastoma by using a highly sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Amplification of tissue-specific mRNA transcript of tyrosine hydroxylase gene with nested primers enabled the detection of residual neuroblastoma cells with a sensitivity of one malignant-cell per 10^6 normals.

Results. Using this method, contaminating tumor cells were detected in seven of nine leukapheresis products of the patients. After positive immunoselection of CD34+ cells on Ceprate™ column, only one of nine enriched stem cell fraction still contained tumor cells detectable by RT-PCR. In six cases, PCR positive PBSCs became PCR negative after selection.

Conclusions. We conclude that tumor cell contamination may be frequently detected in PBSC harvests of stage IV neuroblastoma patients by sensitive molecular analysis. The load of contaminating malignant cells might be reduced following CD34 selection. Med. Pediatr. Oncol. 30:228–232, 1998.

Key words: neuroblastoma; peripheral blood stem cells; tumor cell contamination; CD34 cell selection; tyrosine hydroxylase; RT-PCR

INTRODUCTION

Neuroblastoma is the most frequent extracranial solid tumor in children. Approximately 60% of neuroblastoma patients have advanced stage IV disease at diagnosis with bone marrow (BM), bone, and lymph node metastases [1]. High-dose chemotherapy with autologous hematopoietic progenitor cell support may improve survival in these children [2]. One of the limitations of autologous BM and peripheral blood stem cell (PBSC) transplantations in patients with advanced neuroblastoma is potential tumor cell contamination. Recent studies provide evidence that residual neuroblastoma cells in autografts are highly clonogenic and might contribute to relapse [3,4]. Therefore, some centers prefer the use of PBSC enrichment via positive CD34+ cell selection to reduce the risk of re-infusing malignant cells [5–7]. Determination of purging efficiency of this procedure requires sensitive and reliable methods for accurate detection of residual neuroblastoma cells. These methods are based on immunocytologic assay [8] or reverse transcriptase-polymerase chain reaction (RT-PCR) analysis [9,10]. The RT-PCR amplification of tyrosine hydroxylase (TH)

Contract grant sponsor: Ligue Nationale contre le Cancer, Comités du Puy-de-Dôme et de la Creuse.

[1]Unité Bioclinique de Prélèvement et de Traitement des Cellules Souches Hématopoïétiques et Secteur d'Etude en Hématopoïèse, Service de Pédiatrie B, France.

[2]Laboratoire de Cytogénétique Médicale, Faculté de Médecine, Clermont-Ferrand, France.

[3]Service de Pédiatrie I, Hôpital Civil, Strasbourg, France.

[4]Service de Médecine Infantile, CHR, Rennes, France.

[5]Département de Médecine et Réanimation Pédiatriques, Hôpital de la Tronche, Grenoble, France.

[6]Service de Pédiatrie et Génétique Médicale, Hôpital Charles Nicolle, Rouen, France.

[7]Service de Pédiatrie, Hôpital Nord, Saint Etienne, France.

[8]Unité Fonctionnelle de Greffe Médullaire, Centre Léon Bérard, Lyon, France.

[9]Service de Médecine Infantile, Hôpital d'Enfants de Brabois, Nancy, France.

*Correspondence to: Dr. Andrei Tchirkov, Laboratoire de Cytogénétique Médicale, Faculté de Médecine, Place Henri Dunant—BP 38, 63001 Clermont-Ferrand Cedex, France.

Received 12 June 1997; Accepted 10 December 1997

mRNA, a tissue-specific marker of neuroblasts, appears to be the most sensitive detection test [9]. Using this technique, we examined PBSC harvests of patients with advanced neuroblastoma before and after CD34+ enrichment to assess tumor cell contamination and, by implication, evaluate the CD34 selection procedure with respect to the capacity for tumor cell purging.

MATERIALS AND METHODS

Patients and Samples

Seven patients with stage IV neuroblastoma who were candidates for high-dose chemotherapy and autologous PBSC transplantation were included in this study (Table I). The diagnosis and stage of neuroblastoma were established by standard cytohistologic analysis of tumor type and/or demonstration of tumor cells in the BM of patients with elevated urinary cathecholamine metabolites, and body scanning with meta-iodobenzylguanidine (MIBG). Before mobilization of PBSCs, patients were treated with two to six cycles of cyclophosphamide, doxorubicin, etoposide, cisplatin, and carboplatin. Four patients underwent surgical resection of the primary tumor. At the time of harvesting, all patients were in BM remission as evaluated by cytohistologic analysis of 10 BM aspirates and two iliac biopsy trephines. Collections of PBSCs were performed on a Cobe Spectra separator (Cobe, Lakewood, CO) as described elsewhere [6,7]. CD34+ cells were positively selected from leukapheresis products (LP) by immunoabsorption columns (Ceprate™ systems, CellPro, Bothell, WA). After selection, the purity of the CD34+ cell fraction was 54% (range, 21–82%) with 95.7-fold (range, 35–250) enrichment [7].

For RT-PCR analysis, 2×10^6 cells were obtained from LP and CD34-selected fractions. Positive controls included a neuroblastoma cell line (IMR-32), and BM samples from patients with newly diagnosed disseminated neuroblastoma with a high percentage of tumor cells in their marrow aspirates. Normal BM ($n = 5$) and PB ($n = 5$) samples from healthy donors served as negative controls. Other negative controls included samples obtained from PBSC harvests of 11 children with non-neuroblastoma malignancies (five had leukemia, one had lymphoma, three had Ewing sarcoma, one had Hodgkin disease, and one had medulloblastoma). Informed consent was obtained from all patients and/or their parents.

RT-PCR

Total RNA was extracted using RNAPlus™ reagent (Bioprobe Systems, Montreuil, France) following the manufacturer's instructions. Before reverse transcription, RNA was treated by DNAse (Gibco BRL, Eragny, France) to remove any contaminating DNA. Five μg of this RNA were reverse transcribed using SuperScript reverse transcriptase (Gibco) in a total volume of 20 μl. Five μl of the RT product was subjected to nested PCR amplification. The first round of PCR was performed with *TH* specific primers from exons 5 and 8 as described by Burchill et al. [9]. For the second round, we designed the inner primer pair TH5 (5′-TGACCTGGACTTGGACCACC-3′, exon 5) and TH7 (5′-CAATCTCCTCGGCGGTGTAC-3′, exon 7), yielding a small and easily detectable amplification product. The reactions were performed using Taq DNA polymerase (Gibco) at 96°C for 30 seconds, 60°C for 30 seconds for 30 cycles followed by a final extension at 72°C for 10 minutes. The second round of PCR was carried out with 1/30 of the first-round product. The integrity of each RNA sample was assessed by a single-round RT-PCR amplification of the *ABL* housekeeping gene mRNA. PCR reactions for *TH* mRNA were performed in quadruplicate. Strict precautions were taken to prevent contamination of samples, and experiments included negative controls from all stages of the reaction.

RESULTS

We screened the PBSCs derived from patients with advanced neuroblastoma in medullary remission for the presence of the *TH* mRNA transcript to detect residual tumor cells before and after CD34 selection. The analysis was based on a nested RT-PCR amplification with the sensitivity of one tumor cell in 10^6 normals. To determine the sensitivity of the RT-PCR assay, serial dilutions of IMR-32 neuroblastoma cells in normal PB and BM cells from healthy donors were performed. The results of a seeding experiment diluting tumor cells in normal PB cells are displayed in Figure 1. In addition, none of the normal blood and marrow samples analyzed showed occasional transcription of *TH* under described PCR conditions. There were no *TH* mRNA transcripts in the specimens of PBSCs obtained from 11 patients with non-neuroblastoma tumors.

Analysis of *TH* mRNA expression yielded positive results in the majority of LP samples (7/9), indicating tumor cell contamination (Table I). In contrast, only one of corresponding purified CD34+ cell fractions was PCR positive. In six harvests with initially detected neuroblasts, no contamination was found after CD34 selection (Fig. 2). This suggests that positive immunoselection reduced the level of contaminating tumor cells below the limit of PCR detection.

DISCUSSION

Patients with advanced neuroblastoma are currently being treated by high-dose chemotherapy followed by autologous PBSC transplantation. Several studies have been performed to evaluate the potential for PBSCs to contain contaminating neuroblastoma cells. Moss et al. [3,8] demonstrated the common presence of circulating

TABLE I. Patients' Characteristics and RT-PCR Results in PBSC Harvests

Patient number	Age (mo)/ Sex	Primary tumor	Metastatic sites	Time of PBSC harvesting (mo after diagnosis)	Residual disease at harvesting	*TH* mRNA transcript in PBSCs	
						before CD34 selection	after CD34 selection
1	36/M	latero aortic	bone, BM[c]	6	bone (MIBG+)	Negative	Negative
2	60/M	retroperitoneum	bone, BM, LN[d]	4.25	primary tumor, bone (MIBG+)	Positive	Negative
				4.75[a]		Positive	Negative
3	18/M	adrenal	bone, BM	3.5	bone (MIBG+)	Positive	Positive
4	60/M	adrenal	bone, BM, LN	7.5	bone (MIBG+)	Positive	Negative
5	11/F	adrenal	bone, BM, LN	4	primary tumor, bone (MIBG+)	Positive	Negative
6	22/M	adrenal	bone, BM, CNS	6	primary tumor, CNS	Positive	Negative
7	30/M	adrenal	bone, BM, LN	4	primary tumor, bone (MIGB+)	Positive	Negative
				7[b]	bone (MIBG+)	Negative	Negative

[a]Second series of PBSC harvesting performed at identical disease status.
[b]Second series of PBSC harvesting performed after surgical resection of primary tumor and postoperative chemotherapy.
[c]LN = Lymph nodes above diaphragm.
[d]CNS = Central nervous system.

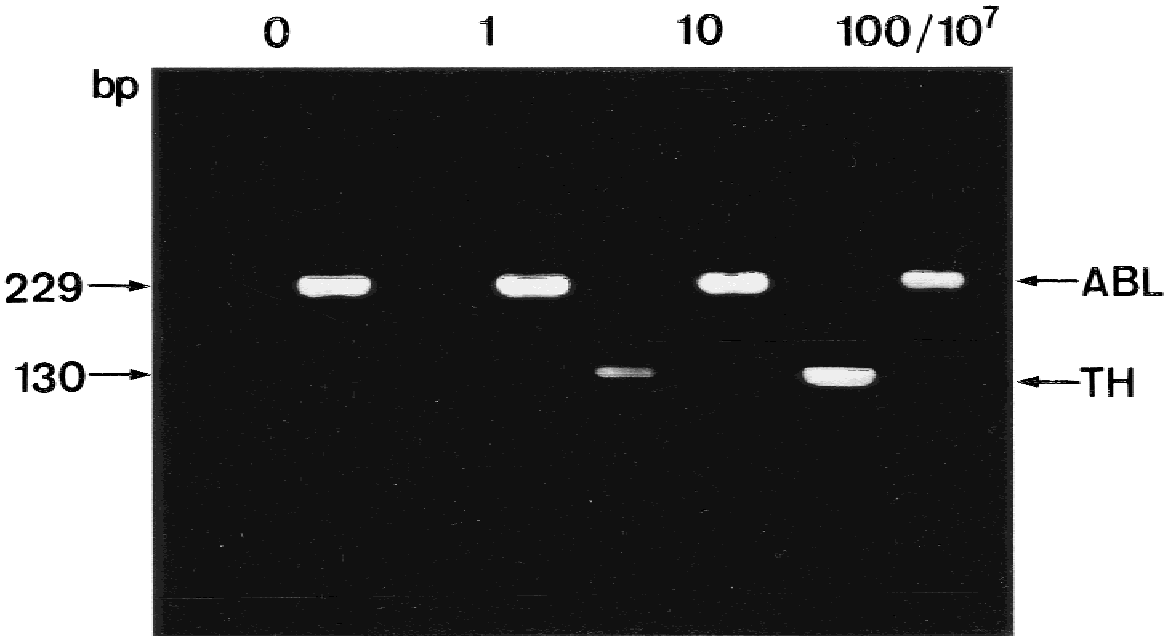

Fig. 1. Serial dilution experiment to determine the sensitivity of nested RT-PCR for *TH* mRNA. Tumor IMR-32 cells were added to peripheral blood cells from a healthy donor to represent 1, 10, and 100 neuroblast(s)/10^7 normal cells. RT-PCR for *TH* mRNA was performed on each of these samples and on a control sample without tumor cell (0). *ABL* mRNA transcript was amplified as a control for the presence of intact mRNA. PCR products were separated by electrophoresis on a 1.8% agarose gel. Tumor cells were detected in the second round of amplification (130 bp product) at as low as 10^{-6} concentration.

neuroblastoma cells in blood samples obtained during and after therapy, and in PBSC harvests by using an immunocytologic assay. Particularly, tumor cell contamination of PBSCs was detected in 25% of patients with disseminated neuroblastoma in medullary remission [8]. By using an RT-PCR for *TH* mRNA, Miyajima et al. [10] found neuroblastoma cells in 20% of PBSC samples and in 66.7% of concurrent BM samples derived from stage IV patients. In our series of neuroblastoma patients evaluated by RT-PCR for *TH* mRNA, we detected residual tumor cells in seven of nine unselected PBSC harvests. Higher tumor cell detection rate in the present study compared to those reported previously might be the result of an increased sensitivity of the nested RT-PCR method. Our *TH* mRNA assay was approximately one order of magnitude more sensitive than the detection techniques used by other investigators [8,10]. Another reason for the high frequency of neuroblastoma cells

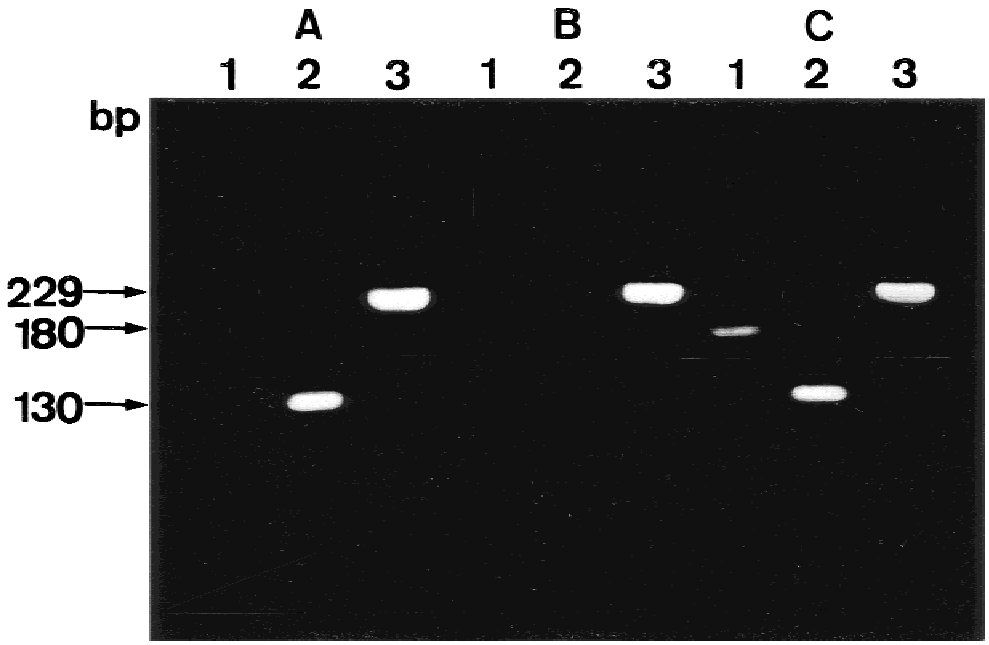

Fig. 2. RT-PCR analysis in LP of patient no. 5 (Table I) before and after CD34 selection. Amplification products obtained after first (1) and second (2) rounds of nested PCR for *TH* mRNA, and a single PCR for *ABL* mRNA, a control of RNA sample quality (3), were separated on a 2.5% agarose gel. **A:** Preselected LP; **B:** CD34+ cell fraction; **C:** Positive control, a $1/10^4$ dilution of neuroblastoma IMR-32 cells in normal blood. LP sample is positive for *TH* mRNA after the second round of PCR. CD34-selected cell sample is negative for *TH* transcript. For positive control sample yielding a weak 180 bp band after the first round of PCR, an aliquot of the first step product was diluted into H_2O at a ratio of 1 to 100 for the re-amplification with nested primers.

within the LP might be that our stage IV patients had a substantial amount of residual tumor at stem cell harvesting. This is consistent with the observation of Miyajima et al. [10], who found tumor cells in PB and LP samples from patients considered to be in partial remission. They reported that all patients with PBSCs negative for *TH* mRNA were in complete remission. Overall, these data indicate that the risk of tumor cell contamination in autologous PBSC transplantation is significant, and rigorous testing of collected stem cells should be performed routinely.

The most common cause of treatment failure in neuroblastoma patients receiving autologous hematopoietic progenitor transplants is disease recurrence. This can be due either to the persistence of residual tumor cell burden following conditioning regimens, or due to the presence of tumor cells in the autologous stem cell harvest used to restore hematopoietic function after megatherapy. The risk of neuroblastoma cell contamination in autologous harvests with respect to tumor relapse is now better understood. Circulating neuroblasts have been shown to be highly clonogenic and, if re-infused, are capable of tumor formation [3]. In order to study the mechanism of relapse after autologous BM transplantations in neuroblastoma, Rill et al. [4] have used retrovirally-mediated transfer of the neomycin-resistance gene to mark harvested BM cells. The introduced gene was detected in malignant cells at the time of relapse, showing that residual neuroblastoma cells in infused marrow made a contribution to disease recurrence. These observations suggest that the application of purging techniques might reduce the probability of relapse after autologous stem cell transplantation in advanced neuroblastoma.

Previously, we have demonstrated the feasibility of immunoselection and transplantation of CD34+ PBSCs in children with advanced neuroblastoma [6,7]. One of the potential benefits of CD34 enrichment of PBSCs is the concomitant depletion of tumor cells. However, the efficiency of neuroblastoma cell purging by this approach was not determined. In the present study, we performed RT-PCR monitoring of tumor cell purging after CD34 selection. Six of the seven LP positive for *TH* mRNA became PCR negative following CD34 enrichment, suggesting that the positive progenitor cell selection procedure was accompanied by indirect tumor cell depletion. Possible explanation for the persistence of tumor cells after CD34 selection in one case (no. 3) might be a high initial neuroblastoma cell load. In fact, this patient relapsed with bone marrow involvement shortly (3 weeks) after leukapheresis. The experimental procedures with an artificial tumor burden in PBSCs should be envisaged in order to analyze a relationship between the initial amount of contaminating tumor cells and the residual tumor contamination after CD34 selection. Combined application of RT-PCR and immunocytologic analyses may be a useful approach for these experiments

to assess the efficiency of tumor cell purging. It will be also of interest to study the additional use of tumor specific antibodies to deplete neuroblastoma cell from PBSCs to clinically satisfactory levels.

In conclusion, the present series shows a high frequency of tumor cell contamination of autologous PBSC harvests obtained from stage IV neuroblastoma patients. By using a sensitive and specific RT-PCR assay for *TH* mRNA, we were able to show that the amount of contaminating residual neuroblastoma cell might be reduced following the CD34 selection procedure. The clinical significance of tumor cell depletion with regard to relapse of disease remains to be determined in these patients.

ACKNOWLEDGMENTS

This study was supported by grants from the Ligue Nationale contre le Cancer, Comités du Puy-de-Dôme et de la Creuse. The authors would like to thank F. Aït-Ouaret for expert technical assistance.

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